Immunity, Vol. 5, 491–501, November, 1996, Copyright ©1996 by Cell Press

Impaired Production and Increased Apoptosis of Neutrophils in Granulocyte Colony-Stimulating Factor Receptor–Deficient Mice

Fulu Liu, Huai Yang Wu, Robin Wesselschmidt, Tad Kornaga, and Daniel C. Link
Division of Bone Marrow Transplantation and Stem Cell Biology
Department of Medicine
Washington University Medical School
St. Louis, Missouri 63110-1093

Summary

We have generated mice carrying a homozygous null mutation in the granulocyte colony-stimulating factor receptor (G-CSFR) gene. G-CSFR-deficient mice have decreased numbers of phenotypically normal circulating neutrophils. Hematopoietic progenitors are decreased in the bone marrow, and the expansion and terminal differentiation of these progenitors into granulocytes is impaired. Neutrophils isolated from G-CSFR-deficient mice have an increased susceptibility to apoptosis, suggesting that the G-CSFR may also regulate neutrophil survival. These data confirm a role for the G-CSFR as a major regulator of granulopoiesis in vivo and provide evidence that the G-CSFR may regulate granulopoiesis by several mechanisms. However, the data also suggest that G-CSFR-independent mechanisms of granulopoiesis must exist.

Introduction

Granulopoiesis is the process whereby (in humans) approximately 120 billion granulocytes are produced daily from a small pool of pluripotent stem cells. Granulopoiesis is a regulated process; its productive capacity can be increased at least 10-fold in response to certain stress conditions such as infection. Granulocyte colony-stimulating factor (G-CSF), a polypeptide growth factor, appears to play a major role in regulating both basal and stress granulopoiesis (reviewed by Demetri and Griffin, 1991). The biological effects of G-CSF are thought to be mediated through its interaction with the granulocyte colony-stimulating factor receptor (G-CSFR), a member of the cytokine receptor superfamily. Indeed, mutations of the G-CSFR have been implicated in the etiology of a subset of severe combined congenital neutropenia (Dong et al., 1995).

The ability of G-CSF to stimulate granulopoiesis is well established (Chatta et al., 1994). Multiple actions of G-CSF have been described that may contribute to the neutrophilic response. First, G-CSF stimulates the proliferation of granulocytic precursors (Souza et al., 1986). Second, it reduces the average transit time through the granulocytic compartment (Lord et al., 1989; Souza et al., 1986). Finally, G-CSF may prolong neutrophil survival (Colotta et al., 1992; Rex et al., 1995). The importance of G-CSF to in vivo granulopoiesis was recently demonstrated in mice carrying a homozygous null mutation for G-CSF; these mice had approximately 20% of normal circulating neutrophils and a corresponding decrease in granulocytic precursors in their bone marrow (Lieschke et al., 1994).

In addition to its effect on granulopoiesis, G-CSF may also contribute to the regulation of multipotential hematopoietic progenitors. The administration of large doses of G-CSF is associated with a dramatic increase in the levels of hematopoietic stem cells and progenitor cells in the peripheral blood (Bungart et al., 1990; de Haan et al., 1995). In vitro, direct effects of G-CSF on primitive progenitor cells have been demonstrated. G-CSF is able to stimulate the formation of granulocyte/macrophage colonies (CFU-GM) from purified CD34-positive progenitors (Haylock et al., 1992). Furthermore, like interleukin-6 (IL-6), G-CSF exhibits synergistic activity with IL-3 to support murine multipotential blast cell colony formation in cultures of spleen cells from 5-fluorouracil-treated mice (Ikebuchi et al., 1988).

Insights into the role of hematopoietic cytokine receptors in the control of hematopoiesis recently have been provided by analysis of mice carrying targeted null mutations of these receptors. The erythropoietin receptor and thrombopoietin receptor (c-Mpl), which like the G-CSFR are predominantly expressed on cells of a single hematopoietic lineage, play crucial roles in the regulation of the production of mature cells of erythroid or megakaryocyte lineages, respectively. Erythropoietin receptor–deficient mice die in utero secondary to a failure of definitive erythropoiesis. However, they contain normal numbers of committed erythroid BFU-E and CFU-E progenitors, indicating that the erythropoietin receptor is not required for erythroid lineage commitment (Wu et al., 1995). Likewise, thrombopoietin receptor–deficient mice are deficient in megakaryocytes and severely thrombocytopenic, but they also have a marked deficiency in committed megakaryocytic progenitors (Alexander et al., 1996; Gurney et al., 1994).

To define further the role of the G-CSFR in the control of granulopoiesis, we have generated a strain of mice carrying a targeted null mutation in the G-CSFR gene by homologous recombination in embryonic stem (ES) cells. In this report, we show that G-CSFR-deficient mice have decreased levels of phenotypically normal circulating neutrophils. Hematopoietic progenitors are decreased in the bone marrow, and the expansion and terminal differentiation of these progenitors into granulocytes is impaired. Neutrophils isolated from G-CSFR-deficient mice have an increased susceptibility to apoptosis, suggesting that the G-CSFR may regulate neutrophil survival. Our results confirm a role for the G-CSFR as a major regulator of granulopoiesis in vivo and provide evidence that the G-CSFR may regulate granulopoiesis by several mechanisms. In addition, we provide evidence that G-CSF may stimulate monocyte production in a G-CSFR-independent fashion and raise the possibility that a second receptor for G-CSF exists on monocyte precursors.

Results

Targeted Disruption of the Murine G-CSFR Gene

To generate a targeted null mutation in the G-CSFR gene, it was first necessary to clone and characterize

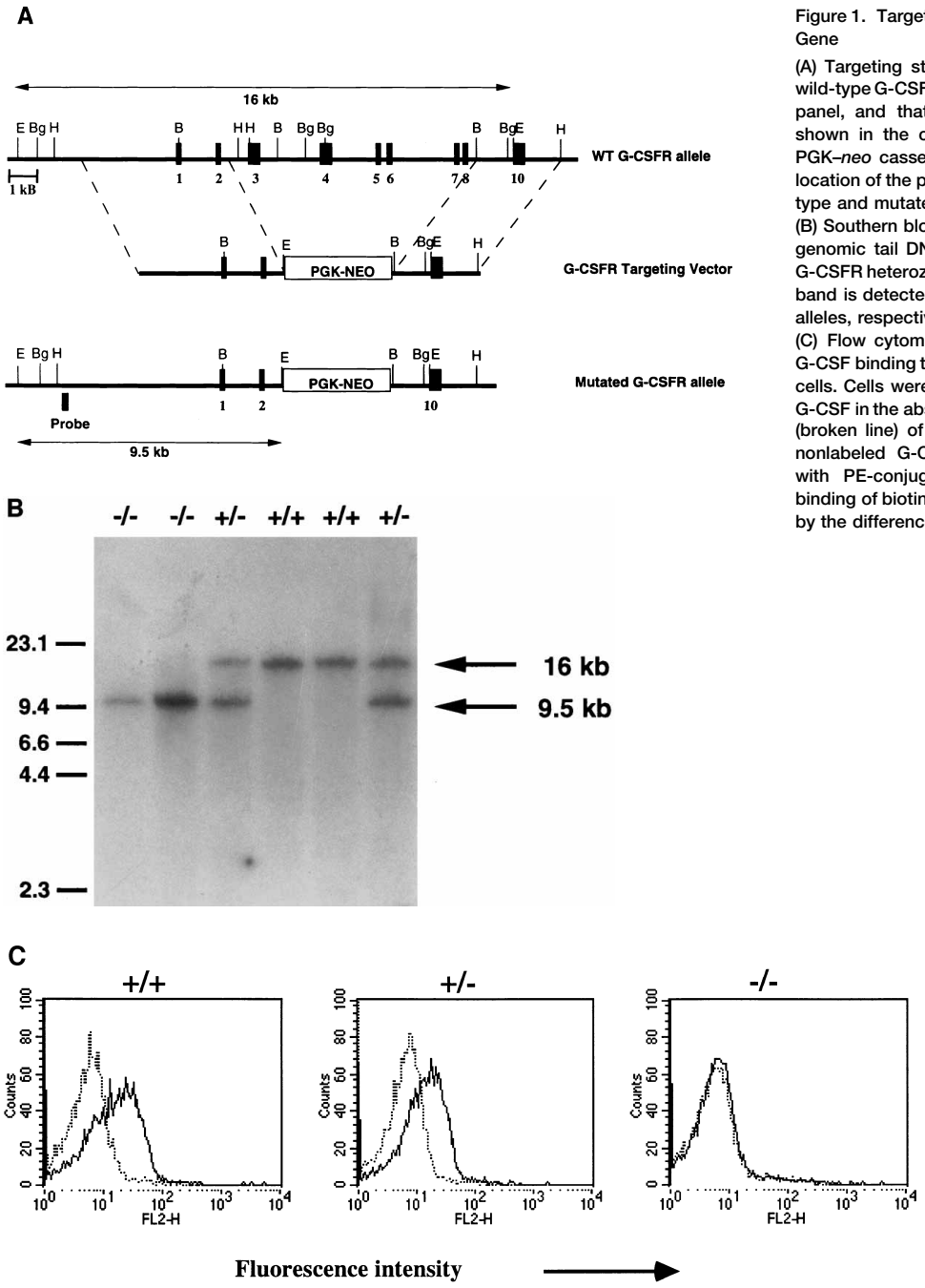

Figure 1. Targeted Disruption of the G-CSFR Gene

(A) Targeting strategy. The structure of the wild-type G-CSFR locus is shown in the upper panel, and that of the targeting vector is shown in the central panel. Note that the PGK–*neo* cassette replaces exons 3–8. The location of the probe used to detect the wild-type and mutated G-CSFR alleles is shown.

(B) Southern blot analysis of EcoRI-digested genomic tail DNA isolated from progeny of G-CSFR heterozygous mice. A 16 kb or 9.5 kb band is detected from wild-type or targeted alleles, respectively.

(C) Flow cytometry analysis of biotinylated G-CSF binding to bone marrow mononuclear cells. Cells were incubated with biotinylated G-CSF in the absence (solid line) or presence (broken line) of a 100-fold molar excess of nonlabeled G-CSF followed by incubation with PE-conjugated streptavidin. Specific binding of biotinylated G-CSF is represented by the difference between the two curves.

the murine G-CSFR gene. We screened a P1 library derived from 129/Ola genomic DNA with primers specific for murine G-CSFR. Three P1 clones were obtained. One clone contained an intronless pseudogene. The other two clones contained overlapping regions of the murine G-CSFR gene. Mapping and sequencing studies were performed to generate the restriction map shown in Figure 1. The murine G-CSFR gene consists of 17 exons (Figure 1A; data not shown); most of the exon–intron boundaries are conserved between the murine and human G-CSFR genes (Seto et al., 1992). The exons were numbered based on the human G-CSFR gene, with the translation initiation codon located within exon 3. We generated a replacement-type targeting vector that contains 4 kb of 5′ targeting sequence, the *neomycin* phosphotransferase gene driven by the phosphoglycerate kinase I gene promoter (PGK–*neo*), and 3 kb of 3′ targeting sequence. Homologous recombination with this vector is expected to replace a 6 kb genomic fragment containing exons 3 through 8 of the G-CSFR with PGK–*neo*. The resulting targeted allele should yield no functional G-CSFR protein, since the translational initiation site, signal peptide, and G-CSF-binding domains of the protein are removed.

RW4 ES cells (Hug et al., 1996) were electroporated, and 240 G418-resistant clones were picked. Southern blot analysis using external and internal probes yielded a single ES clone that contained a targeted mutation of

the G-CSFR gene (data not shown). This ES clone was injected into C57BL/6 blastocysts. Chimeric animals were intercrossed with NIH Swiss Black mice; three of ten chimeric animals tested were able to transmit the G-CSFR mutation through the germline. Progeny from heterozygous intercrosses were genotyped by Southern blot analysis (Figure 1B). The expected numbers of heterozygous and homozygous mutant progeny were obtained. G-CSFR −/− mice develop normally, have normal reproductive ability, and are grossly indistinguishable from their wild-type littermates.

Hematopoietic Cells from G-CSFR-Deficient Mice Express No Functional G-CSFR

The expression of functional G-CSFR on hematopoietic cells was examined using a flow cytometric method to detect specific binding of biotinylated G-CSF. Results are shown in Figure 1C. Specific binding of G-CSF to bone marrow mononuclear cells was readily detected in G-CSFR +/+ animals. As anticipated, no G-CSF binding to G-CSFR −/− leukocytes was detected. Interestingly, G-CSFR +/− animals have an intermediate level of G-CSF binding. Similar results were obtained with peripheral blood leukocytes (data not shown). These data confirm that this targeted mutation of the G-CSFR results in a null phenotype.

G-CSFR-Deficient Mice Have Defective Granulopoiesis

G-CSFR-deficient mice are neutropenic. The results of peripheral blood analysis are shown in Table 1. There was no significant difference in red cell, white cell, or platelet counts between G-CSFR −/− and +/+ mice. However, neutrophil counts in G-CSFR −/− mice were approximately 12% that of +/+ mice. A similar decrease in splenic neutrophils was observed (data not shown), indicating that tissue pools of neutrophils were also reduced. No alterations in lymphocyte or eosinophil counts were observed. Despite the decrease in G-CSF binding observed with leukocytes isolated from G-CSFR +/− mice, neutropenia was not observed in these mice.

G-CSFR −/− mice have decreased numbers of mature myeloid cells in their bone marrow. Examination of hematoxylin–eosin-stained bone marrow sections from G-CSFR −/− mice revealed normal cellularity and megakaryocyte numbers (data not shown). In agreement with this observation, the total number of mononuclear cells recovered from the femurs of G-CSFR −/− mice did not significantly differ from +/+ mice (Table 2). Mature myeloid cells (metamyelocytes and granulocytes) were decreased in G-CSFR −/− mice to approximately 50% that of +/+ mice. Interestingly, no increase in myeloid precursor cells (myeloblasts or promyelocytes) was seen, suggesting that there is no specific block in myeloid differentiation. Erythroid and megakaryocytic development appeared normal.

Table 1. Peripheral Blood Counts

Age (weeks)	G-CSFR Genotype	n	Peripheral Blood Parameter: White Blood Cells (× 10^9/l)	Red Blood Cells (× 10^{12}/l)	Platelet (× 10^9/l)	Percent Polymorphonuclear Neutrophil	Polymorphonuclear Neutrophil (× 10^9/l)	Eosinophil (× 10^9/l)	Lymph (× 10^9/l)	Monocytes[a] (× 10^9/l)
4–5	+/+	6	9.40 ± 7.75	9.34 ± 1.05	940 ± 154	14.0 ± 1.42	1.33 ± 0.36	0.08 ± 0.07	7.86 ± 1.36	ND
4–5	+/−	6	12.48 ± 4.83	8.39 ± 0.48	1019 ± 241	10.8 ± 1.89	1.40 ± 0.74	0.19 ± 0.14	10.72 ± 4.18	ND
4–5	−/−	6	8.33 ± 2.35	8.80 ± 0.79	1051 ± 171	2.1 ± 0.81[b]	0.16 ± 0.03[b]	0.16 ± 0.08	7.79 ± 2.19	ND
10	+/+	10	7.33 ± 2.38	9.39 ± 0.67	910 ± 160	11.6 ± 2.13	0.91 ± 0.24	0.11 ± 0.08	6.61 ± 2.08	0.15 ± 0.05
10	+/−	6	8.12 ± 1.83	9.47 ± 0.71	918 ± 151	9.5 ± 3.74	0.76 ± 0.34	0.19 ± 0.10	7.02 ± 1.59	ND
10	−/−	10	8.82 ± 5.05	8.61 ± 1.25	945 ± 108	1.7 ± 0.63[b]	0.13 ± 0.05[b]	0.06 ± 0.04	8.41 ± 4.78	0.22 ± 0.07[c]
15	+/+	6	9.90 ± 5.48	9.79 ± 0.84	1005 ± 94	14.2 ± 5.84	1.28 ± 0.59	0.25 ± 0.24	8.12 ± 4.65	ND
15	+/−	6	8.80 ± 3.48	10.41 ± 1.00	999 ± 153	15.5 ± 5.22	1.25 ± 0.36	0.23 ± 0.10	7.10 ± 3.26	ND
15	−/−	6	9.33 ± 2.66	9.40 ± 1.42	1173 ± 259	1.2 ± 0.41[b]	0.11 ± 0.05[b]	0.20 ± 0.11	8.89 ± 2.51	ND
28	+/+	5	6.50 ± 3.50	9.46 ± 1.15	1356 ± 218	19.2 ± 9.88[d]	1.26 ± 0.76[c]	ND	ND	0.24 ± 0.14
28	−/−	5	7.04 ± 1.81	9.34 ± 1.34	1319 ± 249	3.5 ± 2.33[d]	0.26 ± 0.20[c]	ND	ND	0.49 ± 0.21[e]

Manual 300-count leukocyte differentials were performed on blood smears from sex-matched littermates. ND, not determined. Data represent the mean ± SD.
[a] Monocyte numbers were determined by flow cytometric enumeration of $CD11b^+/GR\text{-}1^-$ leukocytes.
[b] $p < 0.001$ compared with +/+ mice.
[c] $p < 0.05$ compared with +/+ mice.
[d] $p < 0.01$ compared with +/+ mice.
[e] $P = 0.10$ compared with +/+ mice.

Neutrophil Emigration to Sites of Inflammation and Myeloperoxidase Activity Are Normal in G-CSFR-Deficient Mice

Neutrophil function depends upon both neutrophil development and signaling in the mature neutrophil; both of these processes may be regulated by the G-CSFR. We therefore further examined the phenotype of G-CSFR −/− neutrophils. An important property of neutrophils is their ability to emigrate to sites of inflammation. We examined the extravasation of neutrophils into thioglycollate-inflamed peritoneum. Thioglycollate in-

Table 2. Bone Marrow Analysis

Nucleated Cell Types	Wild Type (Percent ± SD)	Mutant (Percent ± SD)	Probability Value
Total nucleated cells ($\times 10^6$)	33.2 ± 6.4	32.2 ± 7.2	NS
Myeloblasts	0.5 ± 0.4	0.5 ± 0.3	NS
Promyelocytes	1.8 ± 0.6	1.3 ± 0.3	NS
Myelocytes	2.7 ± 1.0	1.9 ± 0.5	NS
Metamyelocytes neutrophil	15.6 ± 1.6	10.6 ± 2.6	0.01
Band and segmented neutrophil	29.6 ± 2.7	14.2 ± 4.1	<0.001
Eosinophil lineage	2.6 ± 0.6	2.8 ± 0.6	NS
Monocytes	0.2 ± 0.1	0.1 ± 0.1	NS
Lymphocytes and plasma cells	6.7 ± 3.4	7.7 ± 3.0	NS
Normoblasts	40.2 ± 6.5	60.9 ± 12	0.01
Myeloid/erythroid ratio	1.3 ± 0.3	0.5 ± 0.2	<0.001

Cell counts and 500-count manual leukocyte differentials were performed on mononuclear cells recovered from both femurs of six sex-matched littermates. Data represent the mean ± SD. NS, not significant.

jection into the peritoneum induces a chemical peritonitis; neutrophils rapidly accumulate in the peritoneum (reaching peak levels at approximately 4 hr) and then are replaced by a primarily monocytic infiltration (Gresham et al., 1991). Neutrophils were readily detected in the peritoneal space of G-CSFR −/− mice 4 hr after injection (Figure 2A). The magnitude of the neutrophil response, although reduced relative to G-CSFR +/+ mice, is appropriate given the overall decreased number of neutrophils present in the periphery and bone marrow of G-CSFR −/− mice.

Myeloperoxidase is a constituent of primary (azurophilic) granules found in myelomonocytic cells. Its expression can be induced in bone marrow mononuclear cells and in 32D cells undergoing myeloid differentiation in response to G-CSF, suggesting that myeloperoxidase expression may normally be regulated by G-CSFR signals (Berliner et al., 1995; Rodel and Link, 1996). We therefore examined myeloperoxidase activity in neutrophils isolated from G-CSFR −/− mice. Neutrophils isolated from the peritoneal space of thioglycollate-treated G-CSFR −/− mice have myeloperoxidase activity indistinguishable from neutrophils isolated from wild-type littermates (Figure 2B).

G-CSFR-Deficient Mice Have Decreased Numbers of Hematopoietic Progenitors

To define the defect in granulopoiesis in G-CSFR −/− mice further, we quantitated hematopoietic progenitors in bone marrow and spleen. As expected, G-CSFR −/−

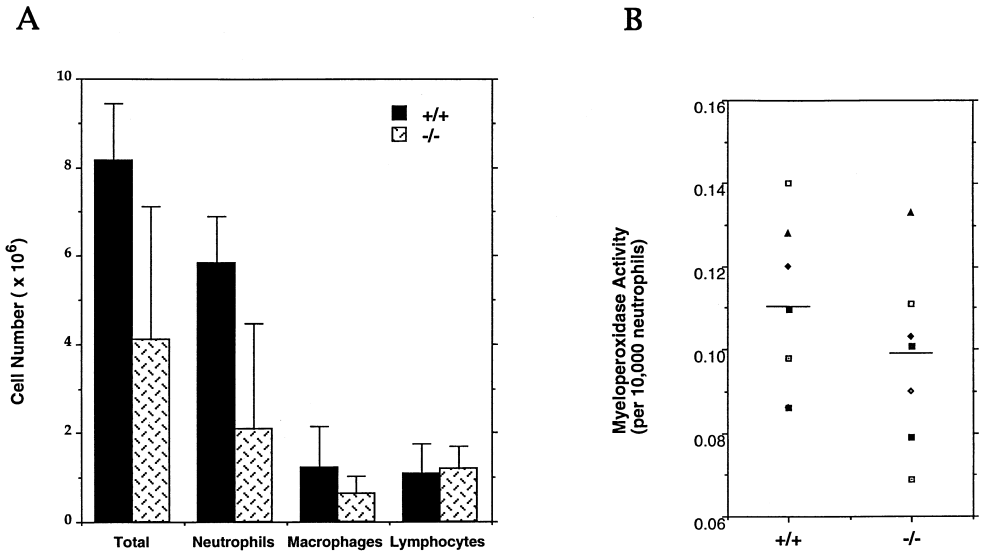

Figure 2. Neutrophil Emigration and Myeloperoxidase Activity

(A) Nucleated cells were recovered from the peritoneal space 4 hr after thioglycollate injection, and 300-count manual leukocyte differentials were performed. Fewer than 0.1 × 10^6 neutrophils were recovered from the peritoneum of control animals (data not shown). Eight animals of each genotype were analyzed. Error bars represent one standard deviation from the mean.

(B) Myeloperoxidase activity. Nucleated cells were isolated from the peritoneum of thioglycollate-injected animals, as described above. Cell lysates were prepared from samples containing equivalent numbers of neutrophils, and the myeloperoxidase activity was assayed using the substrate Azino-bis (3-ethylbenz-thiazoline-6-sufonic acid). The increase in optical density (410 nm) over background per 10^4 neutrophils is shown. No detectable myeloperoxidase activity was observed from cell preparations containing only peritoneal macrophages, lymphocytes, and eosinophils (data not shown). The horizontal bars represent the mean of the data.

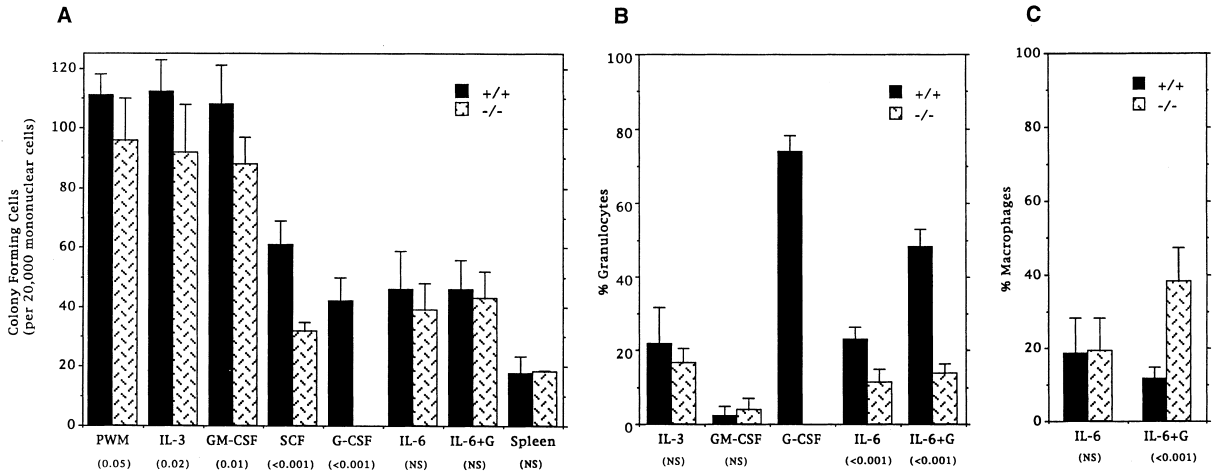

Figure 3. Hematopoietic Progenitor Assays

(A) Bone marrow or spleen mononuclear cells were plated in methylcellulose-containing media supplemented with the indicated cytokine (PWM, pokeweed mitogen–stimulated spleen-conditioned media). Spleen cells were stimulated with PWM. Hematopoietic colonies containing greater than 30 cells were scored after 7–8 days. Seven animals of each genotype were analyzed. Error bars represent one standard deviation from the mean. Probability values are shown at the bottom of the graph.

(B) Granulocyte production in hematopoietic colonies. The percentage of neutrophils was determined by manual 300-count leukocyte differentials on Wright-stained cytospins of cells recovered from entire methylcellulose cultures. Cultures were stimulated with the indicated cytokine for 8 days.

(C) Macrophage production in hematopoietic colonies. The percentage of macrophages was determined as described above. With G-CSFR −/− bone marrow cells, a significant increase in the percentage of macrophages was observed in cultures stimulated with IL-6 plus G-CSF versus IL-6 alone ($p < 0.02$).

bone marrow cells produced no hematopoietic colonies in response to G-CSF (Figure 3A). Relative to G-CSFR +/+ mice, we detected a modest but significant reduction in the total number of hematopoietic colonies formed in response to pokeweed mitogen–stimulated spleen-conditioned media, IL-3, granulocyte/macrophage colony-stimulating factor (GM-CSF), or stem cell factor. These data demonstrate that the G-CSFR is required for the maintenance of a normal number of hematopoietic progenitors.

A Basal G-CSFR-Independent Mechanism for the Production of Mature Granulocytes from Hematopoietic Progenitors Exists

The presence of phenotypically normal neutrophils in G-CSFR-deficient mice suggested that G-CSFR-independent mechanisms of granulopoiesis must exist. To characterize these mechanisms, we examined the ability of hematopoietic progenitors from G-CSFR-deficient mice to produce granulocytes in response to various cytokines. As shown in Figure 3B, mature granulocytes were detected in all cultures regardless of the stimulating cytokine. Interestingly, the percentage of neutrophils produced in cultures stimulated with IL-3 or GM-CSF was similar between −/− and +/+ mice, suggesting that a basal G-CSFR-independent mechanism for the production of mature granulocytes from hematopoietic progenitors exists.

In cultures of both G-CSFR +/+ and −/− hematopoietic progenitors, IL-6-stimulated colonies consisted primarily of granulocytic cells (although IL-6-stimulated colonies contained many immature granulocytic cells; Figure 3B and Figure 4A). We therefore examined the effect of the addition of G-CSF to IL-6 upon hematopoietic colony formation. In cultures of G-CSFR +/+ bone marrow cells, an identical number of hematopoietic colonies formed in response to G-CSF alone, IL-6 alone, or the combination of G-CSF and IL-6, suggesting that G-CSF and IL-6 are stimulating an identical population of hematopoietic progenitors (Figure 3A). The addition of G-CSF did, however, stimulate an increase in colony size and in the percentage of mature neutrophils produced in these colonies (Figure 3B), indicating that G-CSF can stimulate the expansion and terminal differentiation of these committed myeloid progenitors.

Neutrophils from G-CSFR-Deficient Mice Have Increased Susceptibility to Apoptosis

The relative abundance of mature granulocytes in the marrow compared with peripheral circulation in G-CSFR-deficient mice suggested either that neutrophil emigration from the bone marrow was impaired or that neutrophil survival was reduced. G-CSF has been shown to prolong neutrophil survival by suppressing apoptosis (Colotta et al., 1992; Martin et al., 1995; Rex et al., 1995). We therefore examined freshly isolated peripheral blood and bone marrow mononuclear cells for evidence of apoptosis. Apoptosis was assessed by Annexin V binding to surface-expressed phosphatidylserine, a sensitive and early marker of apoptosis (Homburg et al., 1995; Koopman et al., 1994; Martin et al., 1995). As shown in Figure 5, no evidence for a significant population of apoptotic cells was detected in freshly isolated bone

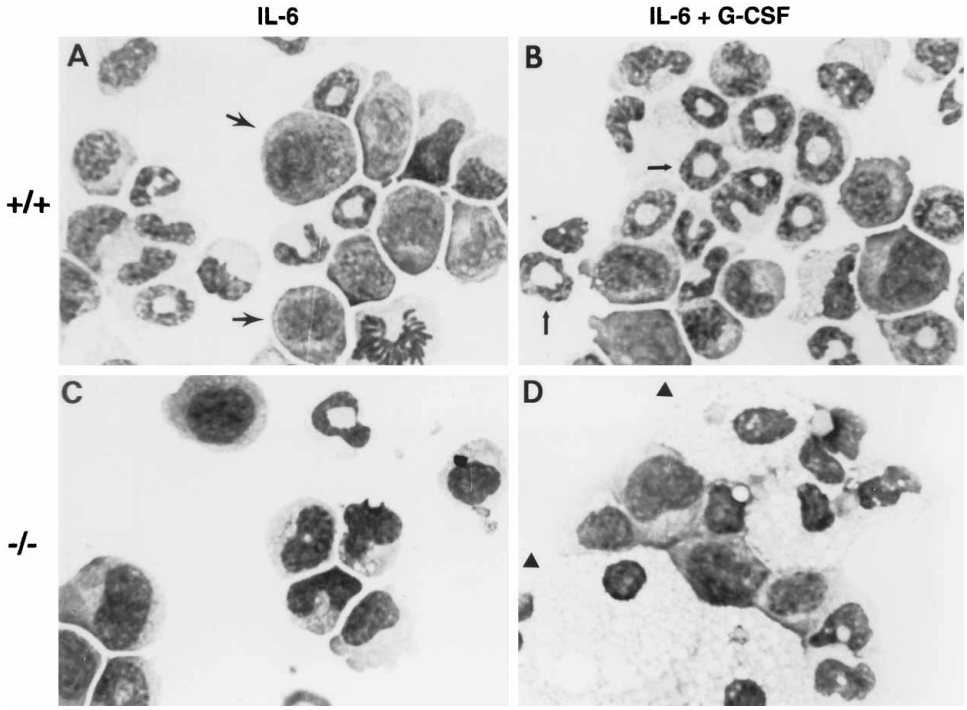

Figure 4. Morphology of Cells Recovered from Hematopoietic Colonies

Wright stain of cells recovered from methylcellulose cultures of G-CSFR +/+ (A and B) or −/− (C and D) progenitors stimulated with the indicated cytokine for 8 days. IL-6-stimulated hematopoietic colonies were made up predominantly of immature myeloid cells (large arrows in [A] and [C]). The addition of G-CSF stimulated the production of mature granulocytes (small arrows) in cultures of G-CSFR +/+ bone marrow cells (B). In contrast, a significant increase in the percentage of macrophages (arrowheads) was detected in hematopoietic colonies generated from G-CSFR −/− progenitors stimulated with G-CSF and IL-6 ([D] and see Figure 3C).

marrow from G-CSFR-deficient animals. However, apoptotic cells may be rapidly cleared in vivo by the reticuloendothelial system. We therefore examined neutrophil survival in vitro. Bone marrow mononuclear cells were cultured in serum-containing media with or without G-CSF. After 48 hr, 31% of Gr-1-positive (myeloid) cells from G-CSFR +/+ mice were apoptotic. In agreement with previous work, the addition of G-CSF to these cultures was able to suppress neutrophil apoptosis substantially. In contrast, in cultures with G-CSFR −/− bone marrow cells, 68% of Gr-1-positive cells were apoptotic at 48 hr, and no response to G-CSF was noted, as expected. The modest increase in the percentage of apoptotic cells in the Gr-1-negative fraction of G-CSFR −/− compared with G-CSFR +/+ cultures suggests that a minority of Gr-1-negative cells may also have a prolonged survival in response to G-CSF. This increased susceptibility to apoptosis is not secondary to mouse strain differences, since a similar percentage of apoptotic cells was observed in cultures of bone marrow cells isolated from Swiss Black or 129/Sv mice (data not shown). These data demonstrate that neutrophils from G-CSFR-deficient mice have an increased susceptibility to apoptosis and suggest that the G-CSFR may play a role in regulating neutrophil survival.

Evidence That G-CSF Can Stimulate Monocyte Production in a G-CSFR-Independent Fashion

In addition to its effects on granulopoiesis, G-CSF is also able to stimulate monocyte production. We quantitated peripheral blood monocytes by flow cytometry ($CD11b^+/GR\text{-}1^-$ cells; see Table 1) and by manual differentials of blood smears (data not shown). In contrast with G-CSF (ligand)-deficient mice, which have peripheral blood monocyte numbers approximately 50% that of wild-type mice, G-CSFR-deficient mice have a relative monocytosis with peripheral blood monocyte numbers approximately 150% that of wild-type mice. We postulated that this phenotypic difference may be secondary to endogenous G-CSF stimulation of monocytopoiesis in G-CSFR-deficient mice. To examine this hypothesis further, we stimulated mice ($n = 8$) with 250 μg/kg per day of human G-CSF for 5 days and measured their leukocyte responses. Wild-type mice had the expected increase in circulating neutrophils (11.6 ± 6.8-fold increase over baseline) and monocytes (2.71 ± 0.99-fold increase over baseline). In contrast, no significant increase in circulating neutrophils (1.36 ± 0.55-fold increase over baseline) or monocytes (1.39 ± 1.07-fold increase over baseline) was detected after G-CSF stimulation of G-CSFR-deficient mice. We reasoned that this lack of response to G-CSF might be due to the chronic stimulation of monocytopoiesis by endogenous G-CSF in these mice. We therefore examined the ability of G-CSF to stimulate monocyte production from G-CSFR −/− hematopoietic progenitors. As noted above, G-CSF and IL-6 appear to stimulate an identical population of hematopoietic progenitors; with G-CSFR +/+ bone marrow cells, the major effect of the addition of G-CSF to IL-6-stimulated cultures is the increased production

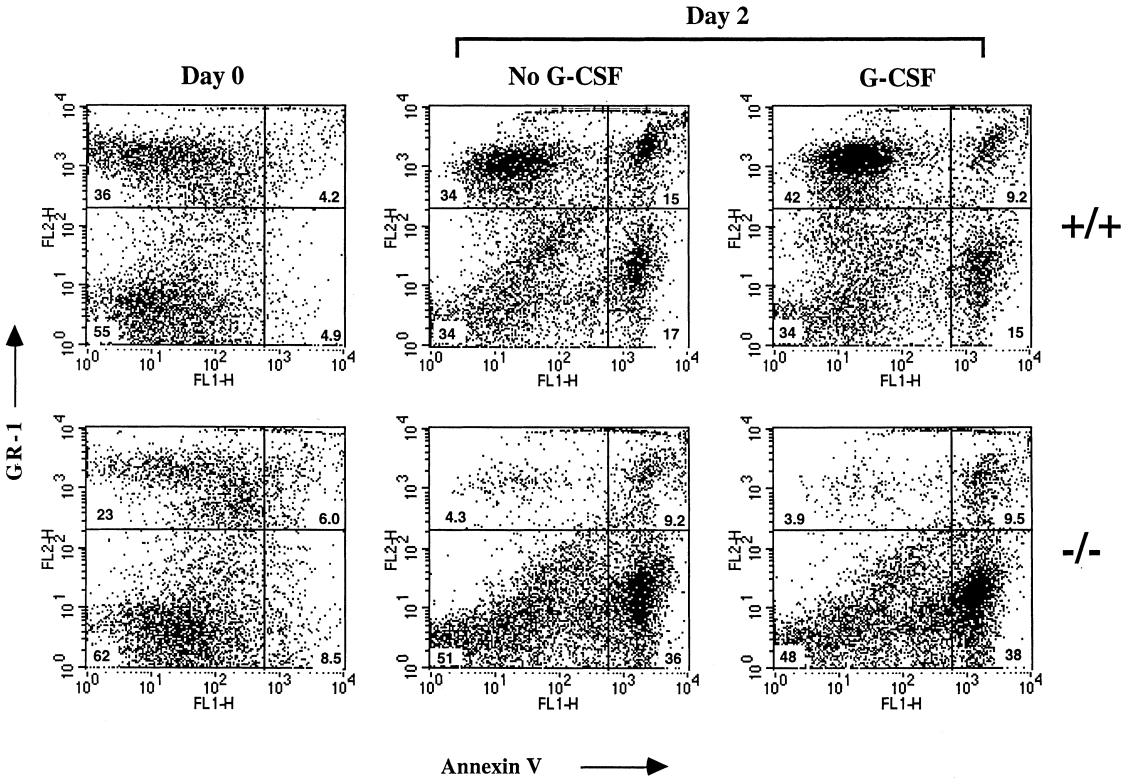

Figure 5. Apoptosis of Neutrophils in Culture

Bone marrow mononuclear cells were cultured in serum-containing media with or without G-CSF for 48 hr. At the indicated time, cells were harvested, stained with Annexin V (an early marker of apoptosis) and Gr-1 (a granulocyte-specific marker), and analyzed by flow cytometry. The percentage of cells present in each quadrant is indicated. With G-CSFR +/+ bone marrow cells, 31% of the Gr-1-positive cells are Annexin V positive (apoptotic) after 48 hr in culture without G-CSF; with G-CSF, this percentage decreased to 18%. In contrast, with G-CSFR −/− bone marrow cells, 68% of the Gr-1-positive cells are Annexin V positive after 48 hr in culture, and no response to G-CSF was observed.

of mature granulocytes. In contrast, the addition of G-CSF to IL-6-stimulated cultures of G-CSFR −/− progenitors reproducibly increased the percentage of macrophages produced in these colonies (Figures 3C and 4), demonstrating that, in vitro, G-CSF can stimulate monocyte production in a G-CSFR-independent fashion.

Discussion

In this study, we have generated homozygous mice carrying a null mutation in the G-CSFR gene. Analysis of G-CSFR-deficient mice confirms the hypothesis in the literature that the G-CSFR is a major regulator of granulopoiesis: G-CSFR-deficient mice have defective granulopoiesis with chronic neutropenia and a decrease in mature myeloid elements in their bone marrow. We provide evidence that the G-CSFR may regulate granulopoiesis by several mechanisms. G-CSFR-deficient mice have decreased numbers of hematopoietic progenitors, and the expansion and terminal differentiation of these progenitors into granulocytes is impaired. Further, we show that neutrophils isolated from G-CSFR-deficient mice have an increased susceptibility to apoptosis, suggesting that the G-CSFR may also regulate neutrophil survival. Finally, we provide evidence that G-CSF may stimulate monocyte production in a G-CSFR-independent fashion.

Severe combined neutropenia (SCN) is a rare congenital disorder manifested by neutropenia and an arrest of myeloid maturation at the promyelocyte or myelocyte stage (Kostmann, 1956). It has been hypothesized that the defect in granulopoiesis observed in this syndrome is secondary to a defect in G-CSFR signaling (Dong et al., 1995). Our data do not support this hypothesis. No accumulation of early myeloid precursors was detected in G-CSFR −/− mice. It remains formally possible that abnormal signaling through the G-CSFR, as opposed to the absence of G-CSFR signaling, could produce the SCN phenotype.

Role of the G-CSFR in Granulopoiesis

The ability of G-CSF to stimulate granulopoiesis is well documented. Multiple mechanisms have been proposed to mediate this effect, including the stimulation of primitive hematopoietic progenitors, proliferation of committed granulocytic precursors, and the facilitation of granulocytic maturation. The biological importance of these activities is not clear. Our study now shows that G-CSFR-deficient mice have a modest reduction in the number of hematopoietic progenitors. In addition to regulating hematopoietic progenitor cell number, the

G-CSFR also appears to regulate the production of mature granulocytes from individual progenitors. The addition of G-CSF to IL-6-stimulated hematopoietic cultures significantly increased the number of mature granulocytes. This effect does not appear to be secondary to the stimulation of additional hematopoietic progenitors, since the total number of colonies formed in response to IL-6 alone, G-CSF alone, and the combination of IL-6 plus G-CSF was not significantly different.

The hypothesis that the G-CSFR transmits granulocytic differentiation signals to hematopoietic progenitors is controversial (reviewed by D'Andrea, 1994). This hypothesis predicts that granulopoiesis in the absence of these signals might lead to functionally defective neutrophils. No such abnormalities were detected in the neutrophils produced in G-CSFR-deficient mice. Neutrophil morphology and surface expression of the myeloid antigens Gr-1 and CD11b were normal (data not shown). In addition, no defect in the ability of neutrophils from G-CSFR $-/-$ to emigrate to sites of inflammation was detected. Myeloperoxidase expression, thought to be linked to G-CSFR signals (Berliner et al., 1995; Rodel and Link, 1996), was also normal in G-CSFR-deficient neutrophils. These neutrophil functional tests are not comprehensive, and significant defects in neutrophil function may have been missed; however, these data provide evidence that the G-CSFR is not required for the development of functionally normal neutrophils. This observation suggests two possibilities: either granulocyte lineage commitment and differentiation is a stochastic process that occurs independently of cytokine signals, or the lineage commitment signals provided by the G-CSFR are redundant.

We show that hematopoietic progenitors from G-CSFR-deficient mice are capable of producing mature granulocytes in culture; in fact, all cytokines tested (with the exception of G-CSF) were able to support the growth and differentiation of granulocytes. This result is consistent with the hypothesis that commitment to the granulocyte lineage is a stochastic process that occurs independently of cytokines. However, it is also possible that a factor(s) produced in common by cells in these cultures is providing alternative signals for granulocyte lineage commitment. Several hematopoietic cytokines apart from G-CSF are known to affect granulopoiesis, and they are potential candidates to provide the necessary granulocyte lineage commitment signals. Mice deficient for both G-CSF and GM-CSF have recently been generated (Seymour et al., 1995). Peripheral blood granulocyte and monocyte counts in these mice were identical to G-CSF-deficient mice, suggesting that GM-CSF is not solely responsible for the residual granulopoiesis in G-CSF-deficient mice. IL-6 is a multifunctional cytokine that has diverse biological effects on hematopoietic cells (Kishimoto et al., 1995). Recent data suggest that IL-6 may play a role in the regulation of granulopoiesis. Although IL-6-deficient mice have normal steady-state granulopoiesis, they are unable to mount a neutrophilic response to Listeria monocytogenes infection (Dalrymple et al., 1995). This observation is consistent with studies in which the administration of recombinant IL-6 to mice induced an increase in peripheral neutrophil counts (Ulich et al., 1989). Interestingly, the G-CSFR and IL-6 receptor complex initiate similar signal transduction cascades (Kishimoto et al., 1995). Indeed, the G-CSFR and gp130 (the signal transduction subunit of the IL-6 receptor complex) share significant homology within their cytoplasmic domains. It is interesting to note that, in the present study, the hematopoietic colonies that formed in response to IL-6 were made up primarily of granulocytic cells. Studies are underway to examine granulopoiesis in mice deficient for both the G-CSFR and IL-6.

The G-CSFR May Regulate Neutrophil Survival In Vivo by Controlling Their Rate of Apoptosis

The ability of cytokines to suppress apoptosis in target cells is well documented (Williams et al., 1990). In particular, G-CSF (along with several other cytokines) is able to suppress apoptosis and prolong neutrophil survival in vitro (Colotta et al., 1992). Recent evidence suggests that this ability may be biologically significant; erythropoietin- and erythropoietin receptor–deficient mice demonstrate increased apoptosis of their erythroid progenitors (Wu et al., 1995). In this study, we show that neutrophils isolated from G-CSFR-deficient mice have an increased susceptibility to apoptosis in vitro. A significant population of apoptotic neutrophils was not detected in freshly isolated bone marrow; however, the rapid clearance of such cells by the reticuloendothelial system may preclude their detection. Two recent reports indicate that the administration of large doses of G-CSF in vivo does not affect neutrophil half-life (Lord et al., 1991; Price et al., 1996). It is possible that baseline levels of endogenous G-CSF are sufficient to suppress neutrophil apoptosis during normal granulopoiesis. Furthermore, G-CSF may play a significant role in regulating neutrophil survival during episodes of infection or other stresses when inflammatory mediators may be affecting neutrophil survival.

G-CSF Stimulates Monocyte Production in a G-CSFR-Independent Fashion

In addition to its effects on granulopoiesis, G-CSF is also able to stimulate monocytopoiesis. In mice, this effect appears to be due to a stimulation of the proliferation of monocyte precursors (Lord et al., 1989). A recent report suggested that the stimulation of monocytopoiesis by G-CSF is indirect and may be mediated by CSF-1 (Gilmore et al., 1995). However, specific (albeit low level) binding of G-CSF to monocyte and monocyte precursors also has been demonstrated (Nicola and Metcalf, 1985). In this study, we demonstrate that the G-CSFR-deficient mice have a resting monocytosis (a typical finding in many patients with neutropenia). In contrast, the G-CSF (ligand)-deficient mice have a resting monocytopenia. It is possible that this difference in monocytopoiesis is due to strain differences in the mice used in these studies (G-CSFR, 129/Sv $\times$ Swiss Black; G-CSF [ligand], 129/Sv $\times$ C57BL/6). However, we detected no difference in baseline levels of circulating monocytes between 129/Sv and Swiss Black mice (data not shown). It is also possible that G-CSF is stimulating monocyte production in the G-CSFR-deficient mice. In

support of this hypothesis, G-CSF in combination with IL-6 was able to stimulate monocyte production from G-CSFR-deficient progenitors. The lack of a significant increase in levels of circulating monocytes in the G-CSFR-deficient mice in response to G-CSF may be secondary to the chronic stimulation of monocytopoiesis by endogenous G-CSF. We are currently working on an assay to measure endogenous G-CSF levels in our G-CSFR-deficient mice. Collectively, these intriguing data suggest that G-CSF may stimulate monocytopoiesis in a G-CSFR-independent fashion and raise the possibility that a second receptor for G-CSF exists on monocyte precursors. Confirmation of this hypothesis will require a definitive demonstration that G-CSF can stimulate monocytopoiesis in vivo in a G-CSFR-independent fashion. To this end, experiments are planned to study monocytopoiesis in mice deficient for both the G-CSF (ligand) and G-CSFR.

In summary, we have confirmed that the G-CSFR is a major regulator of granulopoiesis in vivo. However, the production of phenotypically normal neutrophils in G-CSFR-deficient animals demonstrates that a G-CSFR-independent mechanism(s) of granulopoiesis also exists. Our results indicate that the G-CSFR may regulate granulopoiesis by several mechanisms. First, the G-CSFR appears to play a significant role in the regulation of the production of committed myeloid precursors from more primitive hematopoietic progenitors. Second, it affects the expansion and terminal maturation of committed granulocytic precursors. Finally, the G-CSFR may play a significant role in the regulation of neutrophil survival. In addition, we provide evidence that G-CSF may stimulate monocyte production in a G-CSFR-independent fashion and raise the possibility that a second receptor for G-CSF exists on monocyte precursors.

Experimental Procedures

Construction of G-CSFR Targeting Vector

A P1 genomic library (Genome Systems) from the 129/Ola mouse strain was screened with primers specific for murine G-CSFR: forward primer from exon 5 (5′-CTGCCAGCCCCTCAAACCTATC-3′) and reverse primer from exon 6 (5′-ATGGGGTCGAGGCACAG CTT-3′). Genomic fragments were subcloned into pBluescript (Stratagene). The 4 kb KpnI and 3 kb BamHI–HindIII (blunt, NotI linkered) genomic fragments were inserted 5′ and 3′, respectively, of the 1.8 kb PGK–*neo*^{r} cassette (see Figure 1A). The final 12 kb construct was linearized with NsiI before transfection.

Transfection, Selection of ES Clones, and Southern Blot Analysis

RW4 ES cells (a gift from R. Wesselschmidt and T. J. Ley) were cultured on mitotically inactivated mouse embryonic fibroblasts in standard ES medium. ES cells were electroporated (185 V, 500 μF) with 25 μg of linearized G-CSFR targeting vector and cultured on mouse embryonic fibroblast cells resistant to G418. After 24 hr, the medium was supplemented with 400 μg/ml G418 (GIBCO BRL), and 120 ES clones from each electroporation were isolated after 6–7 days of selection. Individual clones were expanded, and genomic DNA was prepared. DNAs were digested with EcoRI, resolved on 0.8% agarose gels, and transferred to nitrocellulose filters. Filters were hybridized with ^{32}P-labeled probe A (Figure 1B).

Generation of Germline Chimeras

The ES clone containing the null mutation was sent to the University of Cincinnati Medical Center ES cell gene altered mouse service core for injection into C57BL/6 blastocysts. Chimeric animals were intercrossed with NIH Swiss Black mice (Taconic), and germline transmission of the mutant allele was detected by Southern blot analysis of tail DNA from agouti F1 offspring. All mice were housed in a transgenic barrier environment and examined daily by the veterinary staff for signs of illness. A single G-CSFR-deficient mouse developed a pyogenic infection at the site of its ear tag and was euthanized.

Peripheral Blood, Bone Marrow, and Histological Analysis

Blood was obtained by retro-orbital venous plexus sampling in polypropylene tubes containing EDTA. Complete blood counts were determined using a Baker-1000 automated cell counter. Bone marrow was harvested by flushing both femoral bones with 3 ml of α-MEM containing 2% fetal bovine serum (FBS). Spleen cell suspensions were obtained by homogenizing portions of spleen in α-MEM containing 2% FBS. Leukocyte differentials were performed on Wright-stained blood smears or cytospins of purified leukocyte preparations. For histology, formalin-fixed tissues were paraffin embedded, sectioned, and stained with hematoxylin–eosin.

Flow Cytometry

Red blood cells in peripheral blood and bone marrow mononuclear cell preparations were lysed in Tris-buffered ammonium chloride (pH 7.2) buffer and incubated with the indicated antibody at 4°C for 1 hr in PBS containing 0.1% sodium azide and 5% rat serum to block nonspecific binding. The following directly conjugated monoclonal antibodies were used: phycoerythrin (PE)-conjugated rat anti-mouse CDIIb (M1/70, IgG2b; Pharmingen); fluorescein isothiocyanate (FITC)-conjugated rat anti–mouse Gr-1 (RB6-8C5, IgG2b; Pharmingen); PE-conjugated rat IgG2b (R35-38, isotype control; Pharmingen); and FITC-conjugated rat IgG2b (R35-38, isotype control; Pharmingen). To assess surface G-CSFR expression, G-CSF was biotinylated using NHS-LC-biotin (Pierce) as previously described (Shimoda et al., 1992). Peripheral blood mononuclear cells were incubated at 4°C for 1 hr with biotinylated G-CSF (25 ng per 10^6 cells) in the presence or absence of a 100-fold molar excess of nonlabeled G-CSF, followed by incubation with PE-conjugated streptavidin. All cells were analyzed on a FACScan flow cytometer.

Thioglycollate-Induced Peritonitis and Myeloperoxidase Activity

Mice were injected intraperitoneally with 2 ml of thioglycollate broth (DIFCO), and peritoneal lavage was performed 4 hr later. Red blood cells were lysed in Tris-buffered ammonium chloride (pH 7.2) buffer, and leukocytes were enumerated using a Coulter counter (model F) automated counter. Leukocyte differentials were performed on Wright-stained cytospins. Neutrophil myeloperoxidase activity was assayed using these leukocyte preparations. Samples containing equivalent numbers of neutrophils were lysed and assayed for myeloperoxidase activity using the substrate Azino-bis (3-ethylbenzthiazoline-6-sufonic acid) (Sigma) as previously described (Parkos et al., 1991).

Hematopoietic Progenitor Cell Assays

Bone marrow or spleen mononuclear cells were enumerated using a hemacytometer. We plated 2×10^4 bone marrow mononuclear cells or 1×10^5 spleen mononuclear cells in either 1 ml of methylcellulose media (MethoCult M3230, Stem Cell Technologies) supplemented with the indicated cytokines or with 1 ml of methylcellulose media supplemented with erythropoietin and pokeweed mitogen–stimulated murine spleen cell–conditioned medium (MethoCult M3430, Stem Cell Technologies). Colonies were counted on days 7–8. Recombinant cytokines were used at the following concentrations: G-CSF, 10 ng/ml (Amgen); GM-CSF, 10 ng/ml (R&D Systems); IL-3, 10 ng/ml (R&D Systems); IL-6, 500 ng/ml (R&D Systems); and stem cell factor, 100 ng/ml (R&D Systems). To examine cell morphology, entire methylcellulose cultures were harvested and washed extensively to remove the methylcellulose. Leukocyte differentials were performed on Wright-stained cytospins of the hematopoietic cells.

Apoptosis Assay

Bone marrow cells were sterile harvested, as described above, into α-MEM with 5% FBS, 1 mM L-glutamine, and 10 μg/ml ciprofloxacin (Bayer). Cells were cultured in tissue-culture flasks at 37°C in 5% CO_2 for 1 hr to remove adherent cells (including macrophages). The nonadherent cells were harvested and either analyzed as described below or cultured for an additional 48 hr. Where indicated the culture was supplemented with 100 ng/ml G-CSF. For analysis, cells were washed once in α-MEM, once in binding buffer (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 0.8 mM $CaCl_2$, 1 mM $MgSO_4$, 1.2 mM potassium phosphate, 5.5 mM glucose, and 0.5% bovine serum albumin [pH 7.4]) and then resuspended at 1×10^7 cells per milliliter in binding buffer. Cells (10^6) were incubated with FITC-conjugated Annexin V (NeXins Research B. V.) and PE-conjugated rat anti–mouse Gr-1 for 30 min at 4°C, washed twice in binding buffer, and analyzed on a FACScan flow cytometer.

G-CSF Administration to Mice

Recombinant human G-CSF (Amgen) was administered by daily subcutaneous injection at a dose of 250 μg/kg per day for 5 days. Peripheral blood was obtained before the first G-CSF dose and 4–6 hr after the final G-CSF dose. Peripheral blood leukocyte levels were analyzed as described above. A total of eight G-CSFR +/+ and eight G-CSFR −/− mice were studied in two separate experiments. No leukocyte responses were detected in control (saline-injected) mice (data not shown).

Statistical Analysis

Data are presented as mean ± SD. Statistical significance was assessed by Student's t test.

Acknowledgments

Correspondence should be addressed to D. C. L. We thank Nancy Link for her expert technical assistance. We also thank Timothy J. Ley for his critical review of this manuscript. This work was supported by the James S. McDonnell Foundation.

Received April 22, 1996; revised September 19, 1996.

References

Alexander, W.S., Roberts, A.W., Nicola, N.A., Li, R., and Metcalf, D. (1996). Deficiencies in progenitor cells of multiple hematopoietic lineages and defective megakaryocytopoiesis in mice lacking the thrombopoietin receptor c-Mpl. Blood *87*, 2162–2170.

Berliner, N., Hsing, A., Graubert, T., Sigurdsson, F., Zain, M., Bruno, E., and Hoffman, R. (1995). Granulocyte colony-stimulating factor induction of human bone marrow progenitors results in neutrophil-specific gene expression. Blood *85*, 799–803.

Bungart, B., Loeffler, M., Goris, H., Dontje, B., Diehl, V., and Nijhof, W. (1990). Differential effects of recombinant human colony stimulating factor (rh G-CSF) on stem cells in marrow, spleen, and peripheral blood in mice. Br. J. Haematol. *76*, 174–179.

Chatta, G.S., Price, T.H., Allen, R.C., and Dale, D.C. (1994). Effects of *in vivo* recombinant methionyl human granulocyte colony-stimulating factor on the neutrophil response and peripheral blood colony-forming cells in healthy young and elderly adult volunteers. Blood *84*, 2923–2929.

Colotta, F., Re, F., Polentarutti, N., Sozzani, S., and Mantovani, A. (1992). Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. Blood *80*, 2012–2020.

Dalrymple, S.A., Lucian, L.A., Slattery, R., McNeil, T., Aud, D.M., Fuchino, S., Lee, F., and Murray, R. (1995). Interleukin-6-deficient mice are highly susceptible to *Listeria monocytogenes* infection: correlation with inefficient neutrophilia. Infect. Immun. *63*, 2262–2268.

D'Andrea, A.D. (1994). Hematopoietic growth factors and the regulation of differentiative decisions. Curr. Opin. Cell Biol. *6*, 804–808.

de Haan, G., Dontje, B., Engel, C., Loeffler, M., and Nijhof, W. (1995). The kinetics of murine hematopoietic stem cells *in vivo* in response to prolonged increased mature blood cell production induced by granulocyte colony-stimulating factor. Blood *86*, 2986–2992.

Demetri, G.D., and Griffin, J.D. (1991). Granulocyte colony-stimulating factor and its receptor. Blood *78*, 2791–2808.

Dong, F., Brynes, R.K., Tidow, N., Welte, K., Lowenberg, B., and Touw, I.P. (1995). Mutations in the gene for the granulocyte colony-stimulating-factor receptor in patients with acute myeloid leukemia preceded by severe congenital neutropenia. N. Engl. J. Med. *333*, 487–493.

Gilmore, G., DePasquale, D., Fischer, B., and Shadduck, R. (1995). Enhancement of monocytopoiesis by granulocyte colony-stimulating factor: evidence for secondary cytokine effects *in vivo*. Exp. Hematol. *23*, 1319–1323.

Gresham, H.D., Ray, C.J., and O'Sullivan, F.X. (1991). Defective neutrophil function in the autoimmune mouse strain MRL/lpr: potential role of transforming growth factor-β. J. Immunol. *146*, 3911–3921.

Gurney, A.L., Carver-Moore, K., Sauvage, F.J.d., and Moore, M.W. (1994). Thrombocytopenia in c-*mpl*-deficient mice. Science *265*, 1445–1447.

Haylock, D.N., To, L.B., Dowse, T.L., Juttner, C.A., and Simmons, P.J. (1992). *Ex vivo* expansion and maturation of peripheral blood CD34+ cells into the myeloid lineage. Blood *80*, 1405–1412.

Homburg, C.H.E., Haas, M.d., Borne, A.E.G.K.v.d., Verhoeven, A.J., Reutelingsperger, C.P.M., and Roos, D. (1995). Human neutrophils lose their surface FcγRIII and acquire Annexin V binding sites during apoptosis *in vitro*. Blood *85*, 532–540.

Hug, B.A., Wesselschmidt, R.L., Fiering, S., Bender, M.A., Epner, E., Groudine, M., and Ley, T.J. (1996). Analysis of mice containing a targeted deletion of β-globin locus control region 5′ hypersensitive site 3. Mol. Cell. Biol. *16*, 2906–2912.

Ikebuchi, K., Ihle, J.N., Hirai, Y., Wong, G.G., Clark, S.C., and Ogawa, M. (1988). Synergistic factors for stem cell proliferation: further studies of the target stem cells and the mechanism of stimulation by interleukin-1, interleukin-6, and granulocyte colony-stimulating factor. Blood *72*, 2007–2014.

Kishimoto, T., Akira, S., Narazaki, M., and Taga, T. (1995). Interleukin-6 family of cytokines and gp130. Blood *86*, 1243–1254.

Koopman, G., Reutlingsperger, C.P.M., Kuijten, G.A.M., Keehnen, R.M.J., Pals, S.T., and van Oers, M.H.J. (1994). Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood *84*, 1415–1420.

Kostmann, R. (1956). Infantile genetic agranulocytosis: a new recessive lethal disease in man. Acta Paediatr. *105*, 1–78.

Lieschke, G.J., Grail, D., Hodgson, G., Metcalf, D., Stanley, E., Cheers, C., Fowler, K.J., Basu, S., Zhan, Y.F., and Dunn, A.R. (1994). Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. Blood *84*, 1737–1746.

Lord, B.I., Bronchud, M.H., Owens, S., Chang, J., Howell, A., Souza, L., and Dexter, T.M. (1989). The kinetics of human granulopoiesis following treatment with granulocyte colony-stimulating factor *in vivo*. Proc. Natl. Acad. Sci. USA *86*, 9499–9503.

Lord, B.I., Molineux, G., Pojda, Z., Souza, L.M., Mermod, J.-J., and Dexter, T.M. (1991). Myeloid cell kinetics in mice treated with recombinant interleukin-3, granulocyte colony-stimulating factor (CSF), or granulocyte-macrophage CSF *in vivo*. Blood *77*, 2154–2159.

Martin, S., Reutelingsperger, C., McGahon, A., Rader, J., Schie, R.v., LaFace, D., and Green, D. (1995). Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J. Exp. Med. *182*, 1545–1556.

Nicola, N.A., and Metcalf, D. (1985). Binding of I^{125}-labeled granulocyte colony-stimulating factor to normal murine hematopoietic cells. J. Cell. Physiol. *124*, 313–321.

Parkos, C.A., Delp, C., Arnaout, M.A., and Madara, J.L. (1991). Neutrophil migration across a cultured intestinal epithelium. J. Clin. Invest. *88*, 1605–1612.

Price, T.H., Chatta, G.S., and Dale, D.C. (1996). Effect of recombinant

granulocyte colony-stimulating factor on neutrophil kinetics in normal young and elderly humans. Blood *88*, 335–340.

Rex, J.H., Bhalla, S.C., Cohen, D.M., Hester, J.P., Vartivarian, S.E., and Anaissie, E.J. (1995). Protection of human polymorphonuclear leukocyte function from the deleterious effects of isolation, irradiation, and storage by interferon-γ and granulocyte-colony-stimulating factor. Transfusion *35*, 605–611.

Rodel, J.E., and Link, D.C. (1996). Suppression of apoptosis during cytokine deprivation of 32D cells is not sufficient to induce complete granulocytic differentiation. Blood *86*, 858–864.

Seto, Y., Fukunaga, R., and Nagata, S. (1992). Chromosomal gene organization of the human granulocyte colony-stimulating factor receptor. J. Immunol. *148*, 259–266.

Seymour, J.F., Lieschke, G.J., Stanley, E., Grail, D., Quilici, C., Hodgson, G., and Dunn, A.R. (1995). Amyloidosis, hematopoietic and pulmonary abnormalities in mice deficient in both G-CSF and GM-CSF. Blood *86*, 593a.

Shimoda, K., Okamura, S., Harada, N., and Niho, Y. (1992). Detection of the granulocyte colony-stimulating factor receptor using biotinylated granulocyte colony-stimulating factor: presence of granulocyte colony-stimulating factor receptor on CD34-positive hematopoietic progenitor cells. Res. Exp. Med. *192*, 245–255.

Souza, L.M., Boone, T.C., Gabrilove, J., Lai, P.H., Zsebo, K.M., Murdock, D.C., Chazin, V.R., Bruszewski, J., Lu, H., Chen, K.K., Barendt, J., Platzer, E., Moore, M.A.S., Mertelsmann, R., and Welte, K. (1986). Recombinant human granulocyte colony-stimulating factor: effects on normal and leukemic myeloid cells. Science *232*, 61–65.

Ulich, T.R., del Castillo, J., and Guo, K. (1989). *In vivo* hematologic effects of recombinant interleukin-6 on hematopoiesis and circulating numbers of RBCs and WBCs. Blood *73*, 108–110.

Williams, G.T., Smith, C.A., Spooncer, E., Dexter, T.M., and Tayler, D.R. (1990). Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. Nature *343*, 76–79.

Wu, H., Liu, X., Jaenisch, R., and Lodish, H.F. (1995). Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. Cell *83*, 59–67.